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(54) Title: BOVINE SEMEN SEXING

(57) Abstract

A method of separating bovine semen into fractions enriched in either X or Y chromosome bearing sperm, wherein the said bovine semen is subjected to thin layer countercurrent distribution (TLCCD) chromatography using a charge insensitive phase system.

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BOVINE SEMEN SEXING

Field of the invention

This invention relates to a method of producing enriched fractions of bovine semen containing predominantly either X or Y chromosome bearing sperm.

Prior art

There have been many reports of methods to separate X and Y chromosome bearing sperm. These methods are reviewed in Gledhill B. L. (Seminars in Reproductive Endocrinology 6 (1988), 385-395) and include reports of X/Y fractionation on the basis of surface charge differences using electrophoresis (Kaneko, S. et al., Proc. Japan Acad. 59 Ser. B (1983), 276-279) and on the basis of sperm mobility using albumin gradients (Ericsonn R. J. et al., Nature (1973) 253, 421-424). None of these reports have been substantiated and currently no procedure is available to commercially produce enriched X or Y chromosome bearing sperm fractions. Further prior art, relevant only with knowledge of the invention, is discussed separately below.

Summary of the invention

The present invention provides a method of separating bovine semen into fractions enriched in either X or Y chromosome bearing sperm, by subjecting the bovine sperm to thin layer countercurrent distribution chromatography (TLCCD) wherein the two phase aqueous system is a charge insensitive one.

The fractions obtained from the TLCCD form two distinct sub-populations of sperm, one predominantly rich in X chromosome bearing sperm and the other predominantly rich in Y chromosome bearing sperm.

Description of further prior art

Aqueous two-phase partitioning systems are well known to be of use in distinguishing cells on the basis of cell surface characteristics. Using such methods Gauda, A. et al., (Gamete Research 24 (1989), 385-392) have studied differences in rat sperm from the caudal and caput epididymis and the vas deferens. The two-phase partitioning has been improved by using

thin-layer countercurrent distribution (TLCCD) (Walter, H. et al., (1982) Partitioning in Aqueous Two-Phase Systems, New York Academic Press and Sharpe, P. T., Trends Biochem. Sci. (1984) 9 (1), 374-7). Using a TLCCD, a heterogeneous population of cells will be distributed between dextran and polyethylene glycol phases according to a Poisson distribution, where peaks broader than those expected for a purely theoretical homogeneous cell population are an indication of cell surface heterogeneity.

The properties of any given phase system can be altered in a number of ways to allow partition of cells to be determined by different cell surface molecules. Thus, charge-sensitive (CS) phase systems (ones having a potential difference between the two phases - created by the inclusion of high concentration of phosphate ions) partition cells principally on the basis of surface charge molecules. Charge-insensitive (CI) phase systems, i.e. ones in which there is essentially no potential difference between the two phases (created by adding low phosphate and high sodium chloride concentrations), partition cells on the basis of non-charged associated surface properties, often referred to as surface 'hydrophobicity'. When small amounts of PEG, esterified with fatty acids, are included in phase systems, partition is based on surface properties predominantly not detected by CS and CI phase systems. By manipulation of the salt content and the use of fatty acid-esterified PEG, together with adjustment of the polymer concentrations, a range of different phase systems can be produced that partition cells on the basis of different surface characteristics. Walter, H. et al., (supra) cites examples of systems of this kind being used to study heterogeneity among erythrocytes, lymphocytes, osteoblasts, monocytes and many others.

Brief description of the drawings

Figures 1-3 show the distribution of sperm into the different compartments of the TLCCD apparatus displaying the separation into two sub-populations of sperm.

Description of the preferred embodiments

The separation of bovine semen into enriched fractions of either X or Y chromosome bearing sperm is critically dependent on the composition of the two aqueous phases in the TLCCD. Only by carefully controlling the composition of the two phases in the TLCCD apparatus are two distinct sub-populations of bovine sperm obtained. One phase will contain predominantly X chromosome bearing sperm and the other predominantly Y chromosome bearing sperm.

The charge insensitive phase system of use in the present invention preferably comprises two aqueous polymeric phases. These phases preferably comprise dextran and PEG in a percentage ratio (by weight) of from 0.85:1 to 1.06:1 (Dextran:PEG) providing a potential difference between the two phases of 0-2.0mV and an interfacial tension of from 1-20$\mu N.m^{-1}$. The TLCCD may be run at any temperature conducive to the survival of a reasonable proportion of sperm, typically of from 4°C to 25°C. The temperature will influence the preferred ratio of dextran:PEG in the phase system. Thus, at 4°C the preferred ratio is 3.5:4 (0.875:1) and at 18°C it is 4:4 (1:1).

The charge-insensitive phase system will usually additionally include from 0 to 0.5M sodium chloride, from 0 to 0.5M phosphate buffer (pH 7.4) and from 0 to 10% sucrose.

An advantage of the method of the present invention is that the viability of the sperm cells after the separate process is not significantly reduced.

Any TLCCD apparatus comparable to that described by Sharpe, P. T. et al., 1988, Methods of Cell Separation, Amsterdam, Elsevier Science Publication) is suitable for use in the present invention. TLCCDs typically have 60 or 120 compartments. After the countercurrent distribution has been carried out, phosphate buffered saline was added to each compartment and the number of sperm in each compartment counted. Plotting sperm number against compartment number two peaks are observed. The contents of those compartments

contributing to the first peak are pooled to yield a fraction enriched in Y-chromosome bearing sperm. Similarly, the contents of those compartments contributing to the second peak may be pooled to yield a fraction rich in X chromosome bearing sperm.

EXAMPLE

Materials and Methods

Chemicals

Dextran T500 (Batch 01 06905) was obtained from Pharmacia Fine Chemicals, poly (ethylene glycol) 8000 from Sigma Chemical Company and Nycodenz from Nycomed As, Oslo, Norway.

Spermatozoa

Bovine sperm were a gift from the Milk Marketing Board, North Wales. Semen was collected twice weekly and stored in a modification of Reading diluent at 18°C (Revell, 1989, Animal Prod. 48, 579-584).

Partitioning

Phase systems ranging from 3.5% (w/w) dextran and 4% (w/w) poly(ethylene glycol) to 5% (w/w) dextran and 5% (w/w) poly(ethylene glycol) were used. To produce charge-insensitive (CI) phase systems, i.e. ones in which there is essentially no potential difference between the two phases, 25ml 1M NaCl, 10ml 0.5M phosphate (KH_2PO_4/K_2HPO_4) buffer pH 7.4 and 24g sucrose were added per 500g of final mixture. (Johansson 1974 Acta. Chem. Scand. Ser. B. B28, 873-882). The phases were separated and stored at either 4°C or 18°C. Partitioning was either at 4°C or 18°C in a Bioshef Mk5 thin-layer countercurrent distribution apparatus (TLCCD). Cells were partitioned 59 times between the two phases. Each time, cells were shaken with the phases for 30 seconds and the phases were left for 9 minutes to separate. After completion of the countercurrent distribution, phosphate buffered saline was added to convert each fraction into a single phase. The number of cells in each of the 60 fractions was determined using a Coulter Counter. The viability of the spermatozoa after TLCCD was determined using a Nigrosin/eosin (10:1.6%) stain mixture. Cells with non-functional

membranes allow influx of eosin and hence stain pink. Viability is expressed as per cent live (non-pink) sperm.

In some experiments, Nycodenz gradients were used to separate dead sperm cells and debris from the semen mixture. A simple discontinuous gradient was prepared by making stock solutions of 35 and 40% Nycodenz. 10mls of sperm suspension (in Reading diluent) were spun down (2500rpm for 8 mins.) and mixed 50:50 (2mls:2mls) with 40% Nycodenz, then underlayered with 35% Nycodenz (4mls) and spun at 1940rpm for 30 mins. in a bench top centrifuge. A visible band of viable sperm formed at the interface (non-viable sperm form a pellet) was removed using a pasteur pipette, pelleted and resuspended in 0.85mls of upper phase. These cells were loaded on to the TLCCD.

Screening of sperm DNA with Y-chromosome specific probe

Sperm in each compartment of these heterogeneous distributions were analysed for the presence of a Y-chromosome specific sequence by extraction of DNA, followed by blotting and hybridisation with the Y-specific probe, BOV97M (Millar, J. R. et al., Animal Genetics 21 (1990) 77-82). In order to allow for differences in the amount of DNA from each fraction applied to the blots, the same blots were also re-hybridised with an autosomal probe, namely β-actin (sequence available on GENBANK database) and the ratio of Y-specific signal:β-actin signal used as a measure of enrichment of Y-bearing sperm.

Genomic DNA was prepared from fractionated sperm cells, by centrifuging the sperm at 1000rpm for 10 minutes, and resuspending the pellets in 150µl of a solution containing 10mM Tris pH 7.5, 50mM NaCl and 10mM EDTA. The sperm were pre-incubated with 50µl of 140mM β-mercaptoethanol for 20 minutes. 250µg/ml proteinase K and 1% SDS were added and left to incubate at 56°C for 12-18 hours. DNA extraction followed using phenol/chloroform/amyl alcohol (25:24:1) and precipitation using 0.5 volume 7.5M ammonium acetate and two volumes of 100% ethanol. The DNA pellet was dried, resuspended in 50µl double distilled water and stored at 4°C.

A Bio-Dot SF microfiltration unit (Bio-rad) was used to transfer the sperm DNA from each TLCCD fraction, to the hybridisation membrane (Gene Screen Plus). Equal amounts of heat denatured DNA were transferred via a slot blot apparatus under vacuum as described by the manufacturers.

The membranes with bound target DNA were pre-hybridised overnight in 1M NaCl, 1% SDS, 10% dextran sulphate and 2µl/ml denatured sonicated herring sperm. Hybridisation of BOV97M was carried out at 65°C and β-actin at 60°C. An appropriate amount of labelled DNA probe was added to the pre-hybridisation solution and hybridised overnight in a Hybaid hybridisation oven.

The membrane was washed in 2XSSC for 30 minutes at room temperature; two 15 minute washes in 2XSSC, 1% SDS at 65°C for BOV97M and 50°C for β-actin; 0.1XSSC for 30 minutes at room temperature. The membrane was agitated during all hybridisation and washing stages. The membrane was then exposed to X-ray film at -70°C.

All blots were probed first with BOV97M, analysed and then stripped by washing in 0.4M NaOH at 42°C for 30 minutes. After stripping the blots were exposed to X-ray film to ensure complete removal of probe before reprobing with β-actin.

Once the hybridisation results were visualised on autoradiography film the signals were quantified using a scanning densitometer (Shimadzu CS-9000 Dual wave-length flying spot scanner) and a density reading was produced for each sample.

RESULTS

Figures 1 and 2 show the plot of sperm number on the ordinate against compartment (fraction) number on the abscissa for a system utilising a Dextran:PEG ratio 4.5:4 (1.125:1) at 18°C and 4°C respectively. In each Figure two distinct peaks can be observed. Using the material obtained from the experiment shown in Figure 1, the contents of compartments 1-20 were pooled (pool 1) as were those of compartments 21-33 (pool 2). These two pools were screened using the Y chromosome specific probe and the β-actin probe.

The sperm in pool 1 consistently (16 repeats) displayed a high Y-specific:β-actin ratio; 80% of the sperm in pool 1 were calculated to be Y-chromosome bearing. The sperm in pool 2 displayed a lower ratio - only 37% of sperm in pool 2 were calculated to be Y-chromosome bearing.

Thus, pool 1 is described as being a fraction enriched in Y-chromosome bearing sperm and pool 2 a fraction enriched in X-chromosome bearing sperm.

On average only 32% of the total sperm partitioned into peak 1, i.e. peak 1 was smaller than peak 2. Taking this into consideration it was calculated that 46% of the total Y chromosome bearing sperm partitioned into peak 1 and with the remaining 54% into peak 2.

Figure 2 shows the effect of temperature. The less distinct distribution as compared to Figure 1 is due to a change in interfacial tension at 4°C. To compensate a suitable Dextran:PEG ratio to use at 4°C would be 3.5:4 (0.875:1).

Figure 3 shows the effect when the semen was pre-treated by centrifugation with Nycodenz as described above (Dextran:PEG, 4.5:4, 18°C).

Table 1 shows the % viability of the sperm present in each peak in each experiment.

	PEAK 1	PEAK 2
Figure 1	38	62
Figure 2	38	62
Figure 3	54	53

CLAIMS

1. A method of separating bovine semen into fractions enriched in either X or Y chromosome bearing sperm, wherein the said bovine semen is subjected to thin layer countercurrent distribution (TLCCD) chromatography using a charge insensitive phase system.

2. A method according to claim 1, wherein the charge insensitive phase system comprises two aqueous polymeric phases.

3. A method according to claim 2, wherein the two aqueous polymeric phases are dextran and poly(ethylene glycol) (PEG) within the ratio dextran:PEG of from 0.85:1 to 1.06:1 by weight.

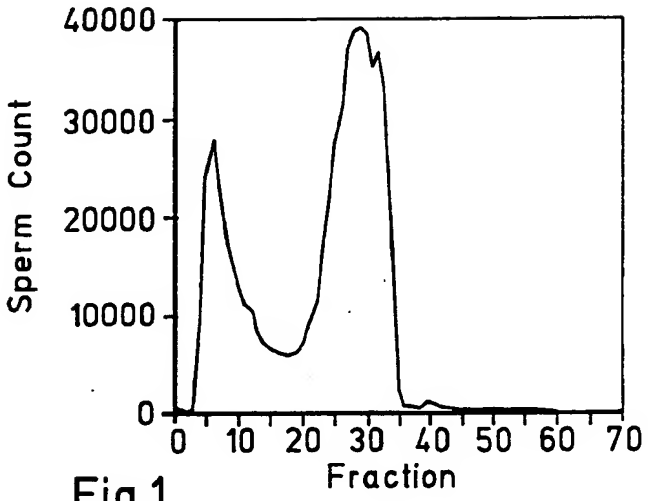

Fig.1

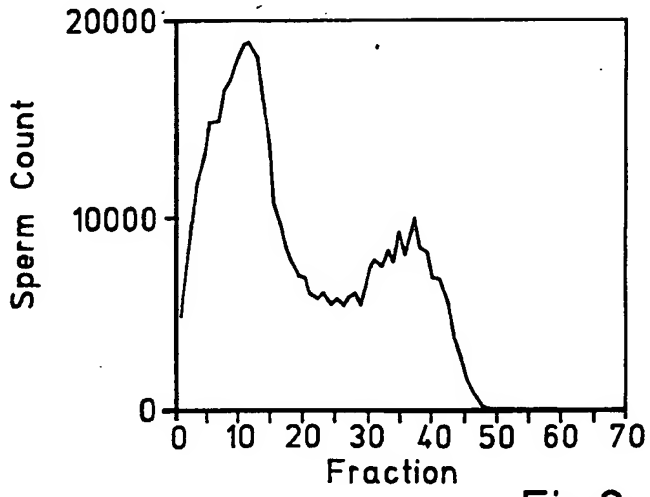

Fig.2

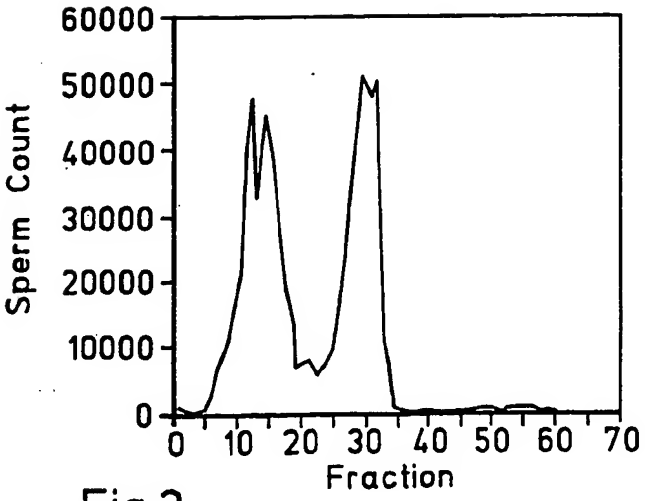

Fig.3

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 92/02187

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A61K35/52

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	US,A,4 722 887 (FABRICANT ET AL) 2 February 1988	1-3
Y	see column 2, line 32 - column 8, line 12 ---	1-3
Y	BIOSCIENCE REPORTS vol. 11, no. 5, October 1991, LONDON,GB pages 265 - 273 CARTWRIGHT ET AL 'SURFACE HETEROGENEITY OF BOVINE SPERM REVEALED BY AQUEOUS TWO-PHASE PARTITION' see page 265, paragraph 1 - page 266, paragraph 7 see page 267, paragraph 2 -paragraph 5 --- -/--	1-3

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
10 FEBRUARY 1993	26.02.93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	SITCH W.D.C.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category°	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	JOURNAL OF CELL SCIENCE vol. 102, May 1992, LONDON,GB pages 123 - 132 HARRISON ET AL 'BEHAVIOUR OF EJACULATED SPERMATOZOA FROM BULL,BOAR AND RAM DURING THIN-LAYER COUNTERCURRENT PARTITION IN AQUEOUS TWO-PHASE SYSTEMS' see the whole document and especially page 123,paragraphs 1-5	1-3
P,Y		1-3
A	SEMINARS IN REPRODUCTIVE ENDOCRINOLOGY vol. 6, no. 4, November 1988, NEW YORK,US pages 385 - 395 GLEDHILL 'GENDER PRESELECTION:HISTORICAL,TECHNICAL,AND ETHICAL PERSPECTIVES' cited in the application see page 388, paragraph 3 - page 392, paragraph 3	1-3
A	WO,A,9 013 315 (CYTOGAM,INC.) 15 November 1990 see page 7, line 29 - page 8, line 3 see page 9, line 10 - line 15 see page 12, line 15 - line 27	1-3

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

GB 9202187
SA 66997

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US-A-4722887	02-02-88	None	
WO-A-9013315	15-11-90	EP-A- 0475936	25-03-92